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13. ABSTRACT *(Maximum 200 words)*

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are genetically and antigenically related viruses with distinct epidemiologic and biologic properties. Since its discovery in West Africa in 1985, various studies have suggested differences between HIV-2 and HIV-1 in geographic distribution, distinct temporal trends in the epidemic spread, and dramatic differences in perinatal and sexual transmission. Studies of HIV-2 infected individuals have shown a significantly slower progression to AIDS. This dramatic difference in pathogenicity provides a unique opportunity to identify viral and host immune mechanisms involved in a closely related virus system that is predicted to have an attenuated phenotype in vivo. This view gave rise to the hypothesis that infection with HIV-2 might provide protection against subsequent infection with the more pathogenic HIV-1. The striking conclusion was that HIV-2 did provide ~60% protection against subsequent infection with HIV-1, now evaluated for over 13 years of study. The "natural experiment" of HIV-2's observed protection against HIV-1 infection represents an invaluable model in which important correlates of HIV-1 protection can be identified and characterized. We are hopeful that further comparative studies of these related immunodeficiency viruses will yield important information on the pathogenic mechanisms employed by HIV viruses and lead the way to the development of effective interventions for the prevention and control of the AIDS pandemic.

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FOREWORD

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Ricki J. Kauli 7/15/99

PI - Signature Date

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Introduction

Human Immunodeficiency Virus Type 2 (HIV-2) was first described in Senegal, West Africa in 1985, by its serologic cross-reactivity to the related simian immunodeficiency virus (SIV) [1]. Subsequent characterization of this new human virus and case reports of associated AIDS cases suggested to some, that a second AIDS epidemic was imminent, this being based on the belief that HIV-2 biology could be readily predicted from our knowledge of HIV-1 [2]. However, almost 15 years since its discovery, research studies conducted both in the laboratory and in HIV-2 infected people have highlighted distinct biological differences between these related viruses [3, 4]. Internationally based epidemiologic and natural history studies of HIV-2 have provided a wealth of biologic data that comprises much of our current appreciation of the unique properties of this related virus. Some of these unique properties include a distinct global distribution of the virus with limited spread, significantly reduced perinatal and sexual transmission, slower rates of progression to AIDS and the potential protective effect of HIV-2 from subsequent HIV-1 infection. Based on our current understanding, the distinct biological differences between these related viruses suggest that viral versus host determinants may be more responsible for the unique pathogenic mechanisms employed by HIV viruses in general. It is hoped that the further characterization of such determinants will be useful for the design of effective HIV interventions.

Geographic distribution of HIV-2

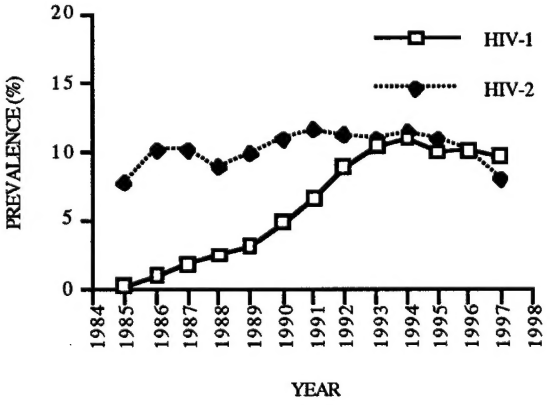
The discovery of HIV-2 in West Africa prompted numerous serologic surveys to further identify its geographic distribution. Over the past decade significant HIV-2 infection has been well documented in most West African countries [5]. Direct comparisons of prevalence rates in different countries are difficult because of differences in study design and diagnostic methodologies; this is particularly pertinent in comparing rates of HIV-dual infections, as described later. A second epidemiologic pattern of HIV-2 infection has been suggested from reports of HIV-2 in Portugal, Mozambique, Angola, southwestern India and Brazil, all areas with former ties to Portugal [6, 7]. Case reports or exceedingly low HIV-2 prevalence rates have been documented in other parts of Africa, Europe, the Americas, the Middle East and Asia, however, its spread has been quite limited. This is further supported by the reduced sexual and perinatal transmission rate of the virus [8-11]. Thus, the current data suggests that HIV-2 has been present in certain populations for a long time in order to establish endemic infection and its spread outside of these endemic areas is limited by a low transmission potential. It therefore seems unlikely that this virus will cause a global pandemic similar to that of HIV-1.

Prevalence and Incidence of HIV-1 and HIV-2 in Dakar, Senegal

In 1970, the Senegalese government established a public health program whereby self-identified sex workers were registered to attend a clinic center, which provides regular evaluation and free treatment for sexually transmitted diseases. The Institut Hygiene Sociale (IHS) clinic in Dakar, Senegal is administered by the Senegalese Ministry of Health. This out-patient clinic is responsible for all the registered sex workers in the Dakar

area. At each clinic visit, women receive extensive counselling on safe sex practices and receive free condoms. These centers, originally managed by social workers and nurse practitioners, were joined by our study physicians in Dakar in 1985, Over the past 13 years we have evaluated this population of high risk women for seroprevalence, seroincidence and identification of risk factors for both HIV-2 and HIV-1 infection [8, 12]. Our ability to prospectively follow these populations has also been demonstrated in a number of natural history studies, in which HIV-2 was found to be less pathogenic than HIV-1 [13], HIV-2 was found to afford protection against HIV-1 [14], and HIV-1 subtype-specific disease progression was found to differ [15]. We had 91.5% passive annual follow up in 1996, which was increased to 96% after active follow-up procedures, resulting in less than 4% loss to follow-up in over 900 women followed. Prevalence data for the past 12 years demonstrates a relative plateau of HIV-2 infection, with HIV-1 surpassing the prevalence of the more endemic virus, HIV-2 (Figure 1) during the observation period.

Figure 1. The annual prevalence of HIV-1 and HIV-2 in commercial sex workers in Dakar, Senegal.



HIV-1/HIV-2 Dual Infection

Since 1986, a number of West African countries have reported significant rates of HIV-1 and HIV-2 infections, in addition, individuals with a HIV-dual serologic profile have been described [16-19]. The HIV dual antibody profile is characterized by antibodies with equally strong reactivity to the *env* antigens of both HIV-1 and HIV-2 by immunoblot and/or radioimmunoprecipitation analysis (RIPA) [1, 19]. A number of explanations for this type of serologic HIV-dual reactivity must be entertained including: extensive cross-reactivity by either of the HIVs, dual infection, infection by one type and exposure to a second type, or infection with an intermediate virus.

Isolation of both HIV-1 and HIV-2 has been reported from select HIV- dual cases [20]; and PCR evidence of HIV-1 and HIV-2 infection has been reported in similar populations . Two studies from the Ivory Coast described 21/34 (61.7%) serologically diagnosed HIV-duals were confirmed by PCR [17], whereas a second report demonstrated 12/36 (33.3%) [21]. We have found that appropriate serological testing and PCR amplification can be highly correlated, particularly when serologic [22] and PCR assays are well standardized and optimized for sensitivity [23]. We have utilized two sets of nested primers for each HIV type with southern blot hybridization to confirm the amplified product. With this methodology we had 100% detection of the appropriate proviral HIV in singly infected individuals and in healthy individuals with HIV dual serologic patterns, all individuals were found to carry both HIV proviruses. By contrast, we found that in serologically designated HIV dual individuals with low CD4+ counts (<400 cells/mm3), PCR confirmation of HIV-2 was compromised [23]. We would hypothesize that when the CD4 cell count decreases the differential viral

replication properties of the two viruses results in an overabundance of cells with HIV-1 provirus. The prospective evaluation of these superinfected individuals with sequential samples will give us a better understanding of the interaction between HIV-1 and HIV-2.

Population biology might also predict that a poorly transmissible and less virulent virus, such as HIV-2, might not perpetuate in populations with significant HIV-1 [24] Although the existence or generation of an intermediate virus in these populations cannot be unequivocably ruled-out, it is apparent that rates of dual-reactivity have been inflated in the past due to sub-optimal specificity of serologic methods employed. Improvement of serologic assays and newer genetic methods for distinguishing the two viruses have improved and this should improve our diagnostic capabilities and our ability to conduct valid studies of HIV-1/HIV-2 interactions.

Natural History of HIV-2 infection

During the late 1980s and early 1990s, natural history studies of HIV-1 infection conducted in the developed world provided important data on the pathogenesis of HIV-1 infection in vivo. Although numerous cross sectional studies of HIV-2 infection were conducted in the late 1980s, they were intrinsically limited in their ability to describe the natural history of HIV-2 infection, which required prospective studies [25]. Studies concerning the natural history of chronic infections such as HIV are difficult to achieve particularly with minimal loss to follow-up; not surprisingly such studies have been rare in developing countries, where viruses such as HIV-2 can be studied.

Our prospective studies conducted in a registered female sex worker cohort in Dakar, Senegal has provided the unique opportunity of measuring the infection and progression rates of both HIV-1 and HIV-2 infections [13, 26, 27]. Kaplan-Meier analysis comparing HIV-2 (n=50) and HIV-1 (n=81) seroincident women were significantly different with HIV-2 infected women demonstrating a slower progression to AIDS (Wilcoxon-Gehan test; p value = 0.006). HIV-1 infected women with known time of infection had a 5 year AIDS-free survival of 66.9%, whereas in HIV-2 infected women the 5 year AIDS-free survival was 94.7% (Figure 2). These differences in survival probabilities between HIV-2 and HIV-1, were also seen for CDC IV disease and CD4+ lymphocyte counts below 400 cells/mm^3 and CD4+ lymphocyte counts below 200 cells/mm^3, as outcomes [13, 27].

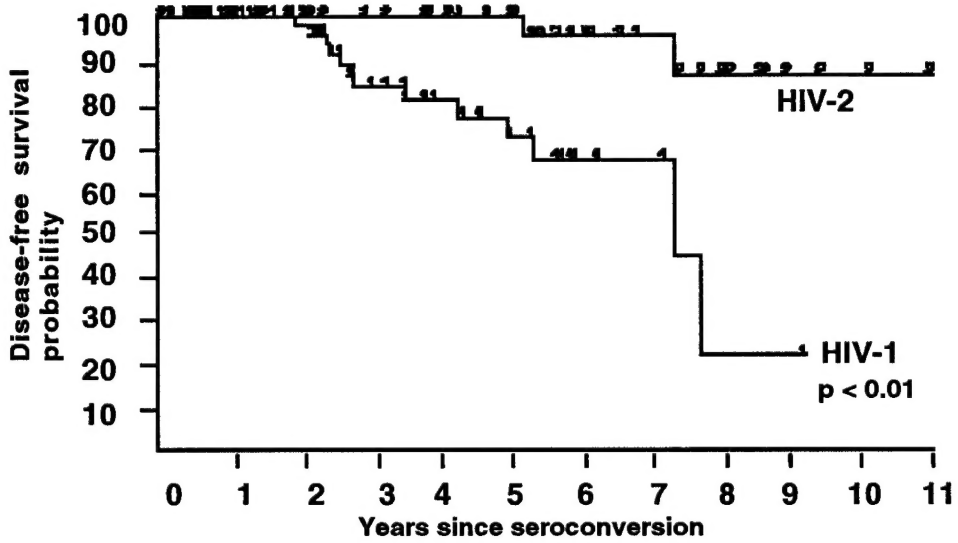


Figure 2: Kaplan-Meier AIDS- free survival probability comparing incident HIV-1 and HIV-2 infected individuals. Wilcoxon-Gehan, p value <0.01.

In our prospective study of HIV-2 infected individuals, we have also identified individuals that fit a definition of long-term non-progression and can determine a rate of this phenotype in the study population. The Kaplan-

Meier analysis of HIV-2 incident infected individuals indicate that 85% (95%CI = 50 - 96%) remain AIDS-free after 8 years of HIV-2 infection. We have also clinically followed a large number of HIV-2 positive prevalent individuals. Recent work by Alcabes et al., indicate that confounding due to differential length-biased sampling in prevalent cohorts does not necessarily bias estimates of the impact of covariates on rates of progression to AIDS. Further, onset bias appears to decrease as study subjects' date of infection becomes more remote [28]. We have therefore combined our HIV-2 prevalent and incident individuals in estimating the rate of long-term non-progression in this virus infection. Using a definition of long-term non-progression of $\geq$8 years infection in the absence of AIDS or related symptoms, and stable CD4+ lymphocytes > 500 cells/mm^3, we have found 39 of 41 (95%) of our women would be classified as long-term non-progressors (Table 1). This dramatic difference in pathogenicity provides a unique opportunity to identify viral and host immune mechanisms involved in a closely related and relevant virus system that is predicted to have a significantly slower course of progression.

Table 1: Long-term progression in HIV-2 Infection

Definition	No. of HIV-2 Positives	No. of LTNPs	Percent LTNPs (%)
>8 yrs symptom free CD4+>500cells/mm^3	41	39	95.1%
>9 yrs symptom free CD4+>500cells/mm^3	22	19	86.4%
>10 yrs symptom free CD4+>500cells/mm^3	13	12	92.3%

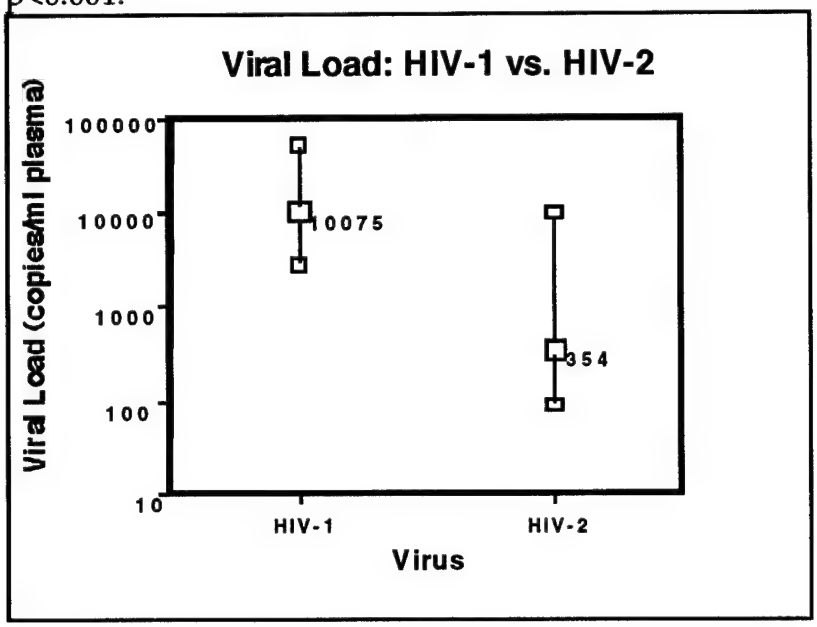
HIV-2 viral dynamics

Plasma viremia has become the standard surrogate marker of HIV progression in the HIV-1 system [29]. Studies of long term non-progressors compared with rapid progressors during the early phases of their infection

have consistently demonstrated lower plasma viral RNA and proviral burdens [30-32]. These individuals have also demonstrated lower seeding of virus in lymphoreticular tissues [33]. In addition, this quantitative assay has demonstrated utility in the SIV system, where plasma viremia at 6 weeks post infection was predictive of disease outcome [34]. Unfortunately to date, a commercial HIV-2 plasma RNA assay is not available.

We have designed a quantitative internally-controlled RT-PCR that amplifies a portion of the gag region of HIV-2, using primers that we have previously shown to be highly sensitive and specific [23]. The assay has a lower limit of detection of 100 copies/ml, and is linear over 4 logs. We determined plasma viral load in individuals from the cohort of registered commercial sex workers in Dakar, Senegal [35]. HIV-2 viral RNA was detectable in 56% of all samples tested; the median load was 141 copies/ml. Levels of viral RNA in the plasma were inversely related to CD4+ cell counts. In a comparison of HIV-2 and HIV-1 viral loads from women in our cohort with known time of infection, we found that the median viral load was 30 times lower in the HIV-2 infected women ($p<0.001$, Wilcoxon rank-sum), irrespective of the length of time infected (Figure 2). This suggests plasma viremia is linked to the differences in the pathogenicity of the two viruses.

Figure 3: The median RNA load of seroconverters to HIV-1 and HIV-2. The error bars represent 25th and 75th percentile. Using the Wilcoxon ranksum test, the median viral loads significantly differ between the two viruses, $p<0.001$.



Although the regulation of viral gene expression in HIV-2 seems to resemble that observed in HIV-1, several differences have been described that may play a role in the differential pathogenicity and in vivo replication of these viruses. Sequence comparisons of HIV-1 and HIV-2 have demonstrated differences in the LTR structure. Whereas HIV-1 has two NF-kB enhancer binding sites, only one can be identified for HIV-2 or most SIVs [36]. The regulation and response to T-cell activation via the viral LTR also appears to be distinct in HIV-2 as compared to HIV-1[37-39]. Specific and unique elements in the HIV-2 LTR may regulate HIV-2 gene expression independently of the T-cell activation signals or cytokines that would normally modulate HIV-1 gene expression [37, 38, 40]. Mutational studies of the unique sites in the HIV-2 LTR responsible for inducible enhancer function demonstrate that this function is more readily disrupted in HIV-2

compared with HIV-1 [40], perhaps explaining some of the distinct biological properties of the virus.

HIV-2 Protection from HIV-1

Given the observations of HIV-2's lower transmissibility [8, 12, 41] and pathogenicity [13, 27] compared to HIV-1, one can easily draw parallels to other systems in which a related less pathogenic virus might induce immune responses that protect against subsequent infection with the more pathogenic virus. This type of interaction can be referred to as a Jennerian approach to vaccination, after Edward Jenner's demonstration that infection with the benign cowpox virus could protect the individual from subsequent infection with the more virulent smallpox virus. In 1995, it was documented in our cohort of commercial sex workers in Dakar, Senegal, that HIV-2 infection conferred a ~70% reduction in the subsequent risk of HIV-1 infection, when controlling for STD infection as a surrogate marker of sexual behavior and immunosuppression as measured by CD4+ counts [14]. We used a Poisson model to estimate the independent effect of demographic, behavioral, and biologic variables on the risk of HIV-1 infection in a cohort of HIV-2 seropositive and HIV seronegative women. Despite higher incidence of other STDs, HIV-2 infected women had lower incidence of HIV-1 than seronegatives, with a incidence rate ratio (IRR) of 0.32 ($p=0.008$). When immunosuppression was accounted for, the IRR associated with HIV-2 seropositivity was reduced further, to 0.23 ($p=0.02$), and the modeling indicated significant effect modification by CD4+ cell count. This analysis led to the conclusion that HIV-2 infection confers a significant reduction in the subsequent risk of HIV-1 infection. This study suggests that the diversity of HIV and SIV viruses previously considered a major stumbling block to

vaccine development may have instead provided a natural model for HIV protection and control.

Continued analysis of the Dakar cohort has extended the observation period from the first published report to over 13 years [42, 43]. These analyses yielded estimates of HIV-2's protective efficacy ranging from 52 to 74%, dependent on the study design [14, 42] suggesting that further unbiased studies of the interaction of the two viruses, controlling for important confounders, are important to determine the generalizability of the noted protective effect (Table 2). It is clear that the protective effect initially noted continues to be documented in this longitudinal study, which is noteworthy for its statistical power generated by the large person-time of observation and narrow confidence intervals.

Table 2. The protective effect of HIV-2 on risk of subsequent HIV-1 infection as documented over time in a cohort of commercial sex workers in Dakar, Senegal. The range of incidence rate ratios (and associated effect measurement, fraction protected) results from inclusion or not of a variable accounting for CD4+ cell count in the Poisson regression model used to estimate the independent effects of potentially confounding variables.

IRR	Fraction Protected	Yrs of Observation	p value
0.23- 0.32	68-77%	9 years	<0.05
0.26-0.36	64-74%	11 years	<0.05
0.33-0.42	58-67%	12 years	<0.03
0.34-0.44	56-66%	13 years	<0.03

Other studies in West Africa have addressed the question of HIV-2 protection from a retrospective analysis. These various studies conducted in different populations and countries have distinct study designs and therefore can only address the question of how generalizable the HIV-2 protection will be in diverse settings [44-46]. Problems with insufficient statistical power, loss to

follow-up and misclassification bias have been previously raised [43] and recent studies report point estimates that fail to achieve statistical significance [46]. Continued scientific discourse around this topic will no doubt continue, but it is hoped that this will be fueled by carefully designed studies that can clearly address this important research topic [42, 43]. Unbiased, powerful studies, using sensitive and specific classification methods, will effectively address the generalizability of the observation of HIV-2's protective efficacy against subsequent HIV-1 infection. Also, they will be able to provide mechanistic insight into the population-based observation of protection. Molecular epidemiologic techniques may identify the host and viral characteristics that interact in those mechanisms.

HIV-2 immunity and correlates of protection

A number of immune mediated host responses might be involved in the in vivo protection described. Available data is supportive of a variety of potential cross-immune effector mechanisms. An early study of MHC-restricted CD8+ CTLs demonstrated HIV-2 gag-specific CTL activity in 5 of 7 HIV-2 infected individuals, in the absence of in vitro restimulation [47]. Studies of cultured CTL responses have shown gag directed activity in 18/20 (90%) and pol directed activity in 14/20 (70%) HIV-2 infected subjects. The sum of specific lysis against HIV-2 gag, pol and nef, or specific lysis of the dominant CTL response, correlated strongly with HIV-2 proviral load [48]. HIV-2 neutralizing antibody activity has also been described in a significant proportion of individuals, the reactivity appears to be broadly reactive and in some cases cross-reactive to HIV-1[49-51] Therefore, in limited studies of HIV-2 specific immunity, there is good evidence for qualitatively superior

responses that are detectable in a larger proportion of individuals, when compared with HIV-1 infected individuals.

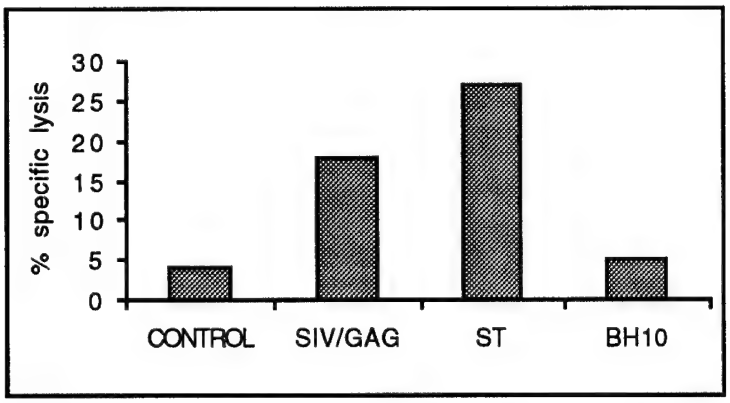


Figure 4: CD8+ cells from subject p1694 were used as effectors with autologous B-LCL infected with recombinant vaccinia virus expressing HIV gene products or control (LAC). BH10=HIV-1 Env, ST=HIV-2 Env. Specific CTL activity was demonstrated against HIV-2 and SIV proteins.

β chemokines have now been identified as potent soluble suppressors of macrophage-tropic HIV infection, in vitro. Studies of multiply exposed uninfected individuals have implicated the role of elevated β–chemokines in HIV resistance, in many cases, independent of genetic mutations in the chemokine receptor [52-54]. Macaque studies have also suggested a role for β-chemokines in vaccine induced protective immunity using a variety of vaccine candidates and live virus challenge [55]. Recently, in vitro observations from our laboratory have suggested similar mechanisms for HIV-2 protection from subsequent HIV-1 infection (Kokkotou and Kanki, unpublished data). Using an in vitro HIV-1 challenge system, we were able to demonstrate that a significant percentage (~60%) of PBMCs derived from HIV-2-infected women could not support replication of a CCR5-dependent HIV-1 virus compared with CXCR4-dependent virus. Resistance was transferable, CD8 dependent and strongly correlated with β–chemokine production in the media. All resistant cultures were rendered susceptible by addition of antibodies to β–chemokines.

HIV-2 infection might dramatically influence β-chemokine production by enhancing it in magnitude and duration, thus enabling HIV-2-infected individuals to cope favorably with subsequent exposure to HIV-1. This is supported by the studies demonstrating that binding of the HIV-2 envelope to the alpha chain of CD8 stimulates dramatic levels of β-chemokine production in comparison to HIV-1 gp120 activity[56]. Not only does this implicate a novel viral suppressive mechanism but one that may be adapted for immunoprophylaxis. Antiretroviral vaccine strategies that incorporate β-chemokine induction or other receptor-blocking functions raise some encouraging possibilities for vaccine design and development .

HIV-1 Cell mediated immunity

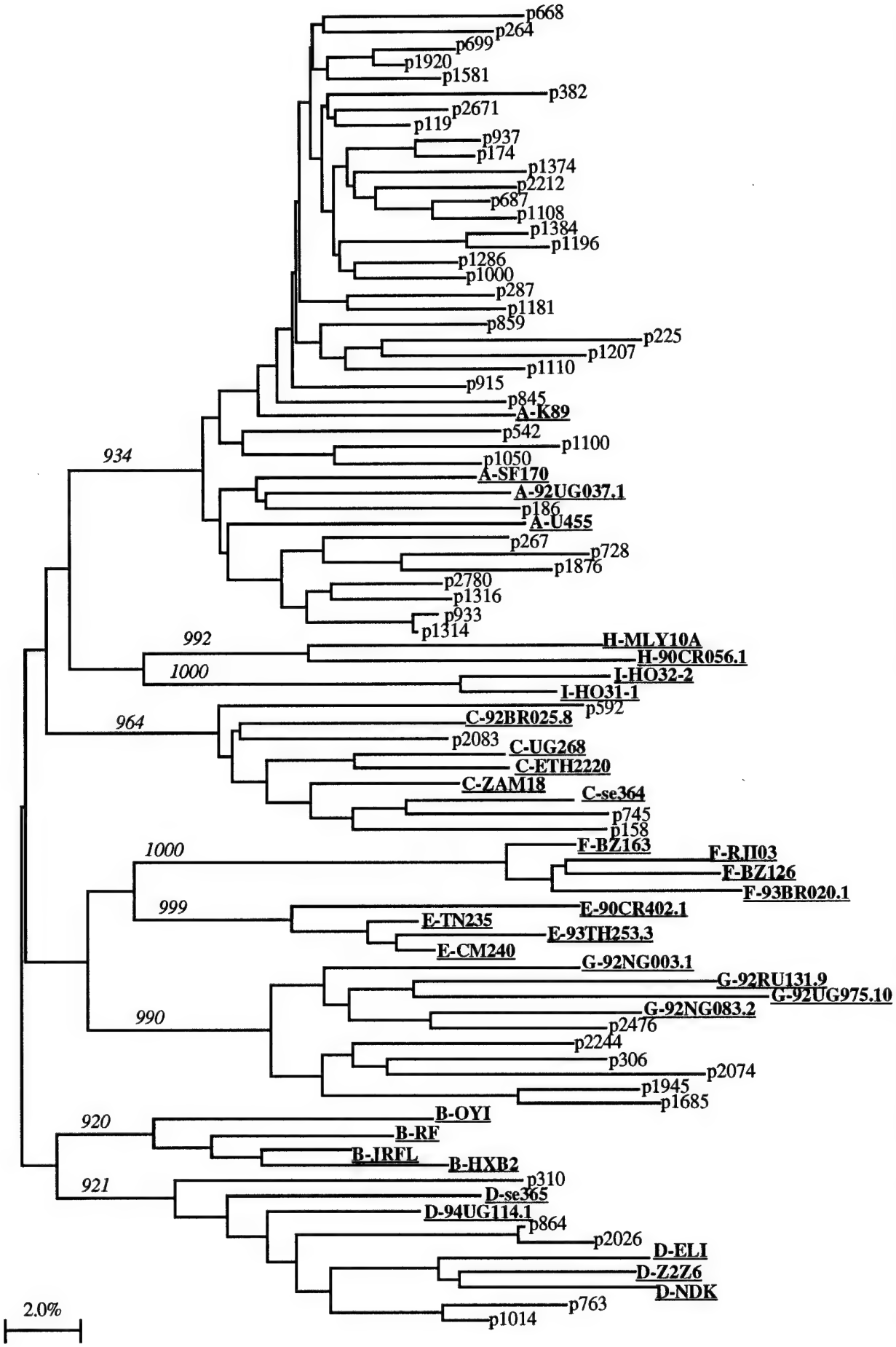
Development of an HIV vaccine has been hampered by concern that viral variation would prevent immunogenic preparations that would protect against a wide variety of HIV strains. To address this concern we undertook a collaborative study between the three sites involved in the present program project submission [57]. This study, aimed at understanding cross-reactive CTL responses between different HIV-1 clades, would be important for the design of a vaccine which would be broadly immunogenic. In this study, we examined the ability of HIV-1 Gag-, reverse transcriptase-, and Env-specific CTL clones isolated from individuals infected in the United States to recognize non-B clade viral sequences and found that all were cross-reactive with the majority of non-B clade viral sequences tested. HIV-1-specific CTL responses from 14 individuals from the cohort in Dakar, Senegal were then analyzed for their cross-reactivity with clade B targets. The cryo-preserved PBMCs were prepared in Dakar and transported to Boston where CTLs assays and sequencing were performed. The individuals were typed by envelope

sequencing and belonged to clade A, C, and G. They all demonstrated cross-reactivity with the U.S. clade B viral expressed targets. This study was among the first to demonstrate that a significant variation in target epitope could still allow immune recognition in HIV infection and that CTL cross-recognition among HIV-1 clades was more frequent than anticipated, thus suggesting that a vaccine based on a single clade might be broadly protective.

Genetic characterization of HIV-1 subtypes [58]

At least 10 different genetic HIV-1 subtypes (A through J) are responsible for the AIDS pandemic. Much of our understanding of HIV-1 disease progression comes from studies in the developed world, where HIV infection is almost exclusively due to subtype B. This has led many to question whether the properties and consequences of HIV-1 infection can be generalized across subtypes that afflict a majority of infected individuals in the developing world. From 1985 to 1997, our prospective study of registered female sex-workers in Senegal has described the introduction and spread of HIV-1 subtypes A, C, D, and G (Figure 5).

Figure 5: HIV-1 subtypes in the Dakar cohort (next page): The neighbor-joining tree performed using Kimura's two-parameter method with 1000 bootstrap replica. Underlined taxa are published sequences from the Los Alamos HIV Database. Letters on the right refer to the different clades.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p915
p845
A-K89
p542
p1100
934
p1050
A-SF170
A-92UG037.1
p186
A-U455
p267
p728
p1876
p2780
p1316
p933
p1314
992
H-MLY10A
H-90CR056.1
1000
I-HO32-2
I-HO31-1
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964
C-92BR025.8
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C-ETH2220
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C-se364
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F-BZ126
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E-90CR402.1
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E-CM240
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G-92UG975.10
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G-92NG083.2
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p1945
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920
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B-IRFL
B-HXB2
921
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D-se365
D-94UG114.1
p864
p2026
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Molecular evolution of HIV-1 subtype A

Subtype A of HIV-1 is the most common subtype in West-Africa. In Dakar, Senegal, it represents about 70% of the HIV-1 infections. We analyzed the temporal evolution of subtype A among a group of female sex-workers with known date of seroconversion over a period of 10 years, from 1988, the first occurrence of HIV-1 subtype A infection in our cohort to 1997. We amplified and sequenced the C2-V3-C3 region of envelope gene from PBMC samples from 48 HIV-1 subtype A infected individuals who seroconverted during the study period. We computed corrected pairwise distances between all available sequences and calculated yearly mean distances defined as the mean of all pairwise distances between sequences of individuals infected the same year (1988 and 1997 had only two infections giving one pairwise distance and were excluded). We observed a temporal diversification of HIV-1 incident infection as the epidemic developed (figure 4). We also calculated the mean pairwise distance for each year relative to the sequences of 1989, and similarly each year relative to the subtype A consensus. In contrast, a relatively flat curve was observed indicating that even with a diversification of circulating strains as the epidemic progress, the variation is confined and radial, as has been described for subtype B [59].

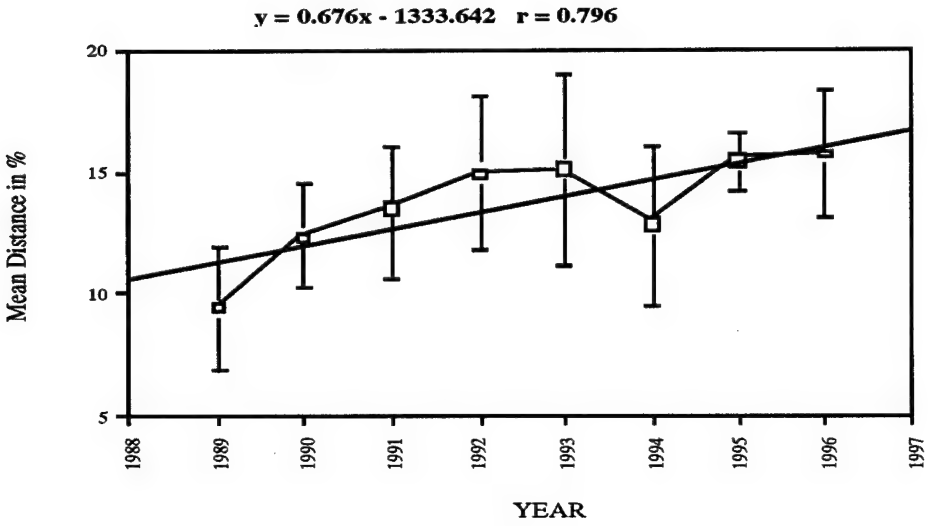

Figure 6: Yearly evolution of HIV-1 subtype A in Senegal: Analysis of the genetic distance revealed a temporal diversification of HIV-1 subtype A incident infection. The mean pairwise distance increased between more homogenous sequences yearly before reaching a plateau in 1995. (Non parametric test for trend, $p < 0.01$).

METHODS

Sample collection and processing

Blood samples were collected on EDTA and transported within 3 hours to the Le Dantec Hospital Laboratory where Ficoll-Hypaque (Organon-Teknika, Durham, NC) separations were performed by a trained biologist. Plasma samples were immediately frozen at -80°C. PBMCs were frozen in media constituted of 90% fetal calf serum and 10% DMSO at a concentration of 2.5 x 10^6 to 1 x 10^7 PBMC/ml. After 16 hours at -80°C, the cells were stored until transport in liquid nitrogen. The cells and the frozen plasma were transported to Boston in the vapor phase of a dry-shipper and re-inserted in liquid nitrogen or at -80°C upon arrival.

DNA was extracted from frozen uncultured PBMCs using proteinase-K phenol extractions. Briefly, the cells were resuspended in sodium chloride-Tris-EDTA/sodium dodecyl sulfate (SDS) 0.5%/ Proteinase-K (100 µg/ml) and

incubated at 56°C for 2 hours. The cell lysates were subjected to four successive phenol-chloroform extractions and ethanol precipitated. The DNA was resuspended in Tris-EDTA and the DNA concentration determined by the optical density at 260 nm.

HIV-2 amplification and sequencing:

A hemi-nested polymerase chain reaction (PCR) was performed to amplify a 525bp fragment spanning the C2, V3, and C3 regions of HIV-2 envelope. Sequences of the primers are:

PK1 : 5'GCGAATTCCCGGGTACTCAAAAGATGT(G,A)(G,A) (6662-6679),

PK2 : 5'GCGAATTCTAGATACTGTGCACCACCGGG (6791-6809), and

PK3 : 5'GCGAATTCGGATCCTCTGCAGTTAGTCCAC (7296-7278).

Primers PK1 and PK3 were used for the first round and PK2 and PK3 for the second round. The primers position are numbered according to the sequence of HIV-2_{ISY} [60, 61]. The PCR reactions were performed in a 100µl volume reaction mixture containing 1 to 2µg of DNA, 10µl of 10X PCR Buffer II (Perkin-Elmer PCR Reagents, Roche Molecular Systems, Branchburg, NJ), 2.5 units of Taq Polymerase (Perkin-Elmer PCR Reagents, Roche Molecular Systems, Branchburg, NJ), 0.2 mM of each dideoxy-nucleotide and 1 to 3 mM of $MgCl_2$ and 20pmoles of each primers. Each reaction was subjected to 35 cycles of denaturation (15 sec. at 94°C), annealing (30 sec. at 47°C and 50°C for the first and second round respectively) and extension (1 min. at 70°C) followed by a final extension of 10 minutes at 70°C in an automatic thermal cycler. Negative controls included deionized water, and either non-infected cell line DNA or negative PBL DNA in all experiments.

The PCR product was purified by agarose gel electrophoresis and direct sequenced using the internal PCR primers. Sequences will be determined by

dye terminator cycle sequencing using Taq polymerase (Perkin-Elmer, Applied Biosystem Division, Foster City, CA) and an automatic sequencer ABI 373 (Perkin-Elmer, Applied Biosystem Division, Foster City, CA).

When necessary, PCR products were cloned in pCRII vector (TOPO T/A cloning, Invitrogen, San-Diego, CA). Positive colonies were chosen based on X-gal metabolism or after PCR screening. Plasmid preparation for double-stranded DNA sequencing was performed by alkaline lysis, using DEAE columns (Qiagen plasmid minikit, Quiagen Inc., Chatsworth, CA) and sequencing performed using vector and insert specific primers.

HIV-1 Amplification and sequencing:

To amplify the HIV-1 V3 region, a nested PCR was performed using two sets of primers:

WT1: 5'GCTGGTTTTGCGATTCTAAAGTGTA (6884-6908, relative to HXB2 [61])

WT2: 5'CAATAGAAAAATTCCCCTCCACAAT (7353-7377), for the first roundand published primers KK30-KK40 [61, 62] for the second round.

The PCR reactions were performed in a 100µl volume reaction mixture containing the DNA, 10µl of 10X PCR Buffer II (Perkin-Elmer PCR Reagents, Roche Molecular Systems, Branchburg, NJ), 2.5 units of Taq Polymerase (Perkin-Elmer PCR Reagents, Roche Molecular Systems, Branchburg, NJ), 0.2 mM of each dideoxy-nucleotide, 20 pmoles of each primer and 2 mM of $MgCl_2$. Each reaction was subjected to 30 cycles of denaturation (15 seconds at 94°C), annealing (30 seconds at 57°C and 55°C for the first and second round respectively) and extension (45 seconds at 72°C) followed by a final extension of 3 minutes at 72°C in an automatic thermal cycler. Negative controls included deionized water, and negative PBL DNA in all experiments.

PCR products processing for sequencing was similar to what is described for HIV-2.

Phylogenetic analysis:

Analysis, alignment and comparison of the sequences obtained were performed with the GeneWorks software (IntelliGenetics, Mountain View, CA) and the Clustal package of multiple alignment programs (Clustal W 1.6) with, as is customary, minor manual adjustment when necessary. Phylogenetic analysis was performed using the Clustal package (Clustal W 1.6) and the Phylip (version 3.5) package [63, 64].

Subtype assignment was based on a neighbor-joining tree with 1000 bootstrap resamplings that include representative of all the major subtypes of HIV-2 or HIV-1 obtained from the HIV Database [65]. To insure the subtype classification, a second phylogenetic strategy was used, generally maximum parsimony [66]. Congruence of results was an indication of an unambiguous subtype assignment.

Conclusions

Since the discovery of the second human immunodeficiency virus in 1985, considerable progress has been made in understanding the virology and epidemiology of HIV-2. The data suggests differences between HIV-2 and HIV-1 in geographic distribution, distinct epidemic trends, differences in perinatal transmission rates and incubation periods to the development of AIDS. The virologic determinants and mechanisms for these apparent biological differences are still unknown. However, an understanding of how HIV-2 differs from HIV-1 is essential for interpretations of comparative virologic studies. We are hopeful that such comparative studies will yield important information on the pathogenic mechanisms employed by HIV

viruses and lead the way to the development of effective interventions for the prevention of AIDS. This is best exemplified in the studies that indicate that this close relative of HIV-1 infection, via its attenuated phenotype, may confer significant protection from subsequent HIV-1 infection. This further suggests that understanding HIV-2 immunity and cross-immunity may be useful for HIV vaccine design and development.

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